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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

518 Rec'd PCT/PTO 24 JUL 2001

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/04140	16 February 2000	18 February 1999

09/890006

TITLE OF INVENTION

PHOSPHOCHOLINE LINKED PRODRUG DERIVATIVES

APPLICANT(S) FOR DO/EO/US

Bruce H. MORIMOTO; Peter L. BARKER

Applicant herewith submits to the United States Designated/Elected office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S. C. 371.

3. [] This is an express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [] A copy of the International Application as filed (35 U.S. C. 371 (c) (2))
   a. [] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [] has been transmitted by the International Bureau
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [] A translation of the International Application into English (35 U.S. C. 371 (c)2)).

7. [] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [] have been transmitted by the International Bureau.
   c. [] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.

8. [] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c) (3)).

9. [X] An (UNSIGNED) oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98 (with 2 references).

12. [] An assignment document for recording. A **separate** cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [] A FIRST preliminary amendment.
    [] A SECOND or SUBSEQUENT preliminary amendment.

14. [] A substitute specification.

15. [] A change of power of attorney an/or address letter.

16. [X] Other items or information: Affirmation of Priority Claim

EXPRESS MAIL CERTIFICATE 903058707-US

Date 7-24-01 Label No EL

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

G Karaszi — G Karaszi

Name (Print) — Signature

JC17 Rec'd PCT/PTO 24 JUL 2001

U.S. APPLICATION NO. (if known see 37 C.F.R.1.50)	INTERNATIONAL APPLICATION NO.: PCT/US00/04140	Attorney's Docket Number
09/890006		

17. [x] The following fees are submitted:		ALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492 (a)(1)-(5)): Search Report has been prepared by the EPO [] or JPO []	**$860.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482)	**$690.00**		
No international preliminary examination fee paid to USPTO(37 CFR 4.482) but international search fee paid to USPTO (37 CFR 1.445 (a) (2)...	**$710.00**		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....	**$1,000.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)....	**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		$100.00	
Surcharge of $130.00 for furnishing the oath or declaration later than []20 []30 months from the earliest claimed priority date (37 CFR 1.492(e)).		$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	**21-20**	1	X $18.00	**$18.00**	
Independent Claims	**6-3**	3	X $80.00	**$240.00**	
Multiple dependent claims(s) (if applicable)		+270		$	
			TOTAL OF ABOVE CALCULATIONS =	**$258.00**	
Reduction by 1/2 for filing by small entity, if applicable.				**$.00**	
			SUBTOTAL =	**$129.00**	
Processing fee of **$130.00** for furnishing the English translation later the [] 20 [] 39 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$	
			TOTAL NATIONAL FEE =	**$129.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). the assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property			+	$0.00	
			TOTAL FEES ENCLOSED =	**$129.00**	
				Amount to be: refunded	$
				charged:	$

a. [X] A check in the amount of $129.00 to cover the above fees is enclosed.

b. [] Please charge my Deposit Account No.04-0100 in the amount of $ to cover the above fees.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0100. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
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REGISTRATION NO. **32,613**

PHOSPHOLIPID DRUG DERIVATIVES

FIELD OF THE INVENTION

This invention pertains to methods and compositions for increasing the aqueous solubility and bio-availability of bioactive agents by conjugating them to phospholipids.

BACKGROUND OF THE INVENTION

Conventional means for delivering pharmaceutical and therapeutic agents to mammals often are severely limited by chemical and physical barriers to uptake, as well as by susceptibility of administered agents to rapid metabolic inactivation following uptake. Oral delivery of many biologically-active agents would be the route of choice if not for the extreme pH of the stomach, the action of proteolytic and other digestive enzymes in the intestine, and the impermeability of gastrointestinal membranes to the active ingredient.

Methods for orally administering vulnerable pharmacological agents have relied on co-administration of adjuvants (e.g. resorcinols and non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether) to artificially increase the permeability of the intestinal walls; co-administration of enzymatic inhibitors (e.g. pancreatic trypsin inhibitor, diisopropylfluorophosphate (DFP) and trasylol) to avoid enzymatic degradation; and encapsulation of the active agent in liposomes or other delivery vehicles.

Irrespective of the mode of administration of many therapeutic compounds, once they gain access to body tissues or fluids they are then subject to rapid inactivation in the liver, termed the first-pass effect. Orally administered compounds in particular are rapidly delivered to the liver via the portal circulation. Many compounds are acted upon by mixed-function oxidases, Phase I enzymes and other liver enzymes to produce inactive glucuronides, hippurates, glycyl and acetyl derivatives, which are rapidly excreted by the kidney.

There is thus a need in the art for methods and compositions to enable potential therapeutic agents to be rapidly absorbed in the intestine and avoid first-pass inactivation in the liver.

SUMMARY OF THE INVENTION

It has now been unexpectedly discovered that conjugation of many biologically active agents to phospholipid via a phosphodiester bond will significantly enhance the bioactivity and/or the bioavailability of such agents.

In one aspect, the present invention provides a method for increasing the bioavailability of a pharmaceutical agent, comprising the steps of conjugating said agent to one or more phospholipid moieties, recovering said biologically active agent conjugated to said phosphocholine and administering said agent to a mammal wherein said agent in conjugated form is significantly more soluble in aqueous media than said agent in unconjugated form.

In yet another aspect, the present invention provides a composition of matter comprising an isolated phospholipid derivative of salicylic acid.

In yet another aspect, the present invention provides a pharmaceutical formulation for treating a mammal suffering from osteoporosis comprising an isolated phospholipid derivative of a compound selected from the group consisting of estrone or estradiol and a pharmaceutically acceptable carrier or diluents.

In yet another aspect, the present invention provides a composition of matter comprising an isolated phospholipid derivative of an antibiotic selected from the group consisting of cephalosporin P1, fusidic acid and helvolic acid.

In yet another aspect, the present invention provides a composition of matter comprising an isolated phospholipid derivative of dehydroepiandosterone.

These and other aspects of the present invention will be apparent to those of ordinary skill in the art in light of the present description, claims and drawings.

DETAILED DESCRIPTION OF THE INVENTION

All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety. In case of inconsistencies, the present description, including definitions, will prevail.

Definitions

"Phospholipid-conjugated" or "phospholipid-derivatized" defined herein as covalently bonded to a phospholipid moiety via a phosphodiester linkage.

"Significantly enhanced bioactivity" or "significantly more soluble in aqueous media" in terms of the conjugated drugs of the present invention is defined herein as no less than 5 to 10-fold increased biological activity and/or aqueous solubility as compared to the unconjugated parent compound when administered by the same route.

The present invention is directed to increasing the bioavailability and/or aqueous solubility of pharmaceutically active agents, specifically by conjugation of such agents to phospholipids, such as a phosphocholine moiety via a phosphodiester bond.

In accordance with the present invention, therapeutic substances will benefit by increasing their water solubility (and their bioavailability) by forming a phosphodiester between an (a) alcohol, and (b) a phospholipid. Non-limiting examples of the phospholipid include phosphocholine, phosphoserine, phosphotyrosine, phosphoethanolamine, n-monoalkyl-phosphoethanolamine and N, N-dialkyl-phosphoethanolamine (all commercially available from Aldrich Chemical, Milwaukee, WI). Phosphocholine is particularly preferred as the phospholipid.

Phosphocholine is a ubiquitous component of biological membranes, usually present in the form of phosphatidyl choline, i.e., attached via a phosphodiester bond to diacyl glycerol. The two most common phosphocholine-containing molecules are lecithin and sphingomyelin. Both of these compounds can be hydrolyzed by phospholipase C at the phosphocholine phosphodiester bond to release diacyl glycerol and ceramides, respectively. Importantly, both lecithin and sphingomyelin, which are present in food, are absorbed in the gastrointestinal tract, incorporated into HDL-and LDL-cholesterol, and transported through the blood without significant first-pass metabolism in the liver.

In accordance with the present invention, conjugation of one or more phospholipid moieties to lipophilic compounds will render them more hydrophilic, without abrogating their ability to traverse biological membranes. Without wishing to be bound by theory, it is contemplated that phospholipid conjugation will, in most cases, mask the biological activity of the conjugated compounds. The phospholipid conjugates will persist in conjugated form until they encounter enzymes such as phospholipase C, sphingomyelinase and non-specific esterases, which are members of the signal transduction

pathway *(Methods in Enzymotofy,* Vol. 197, E. Dennis, editor, Academic Press, NY) and are present in the circulation and on target tissues. These enzymes will then remove the phospholipid moiety and liberate the original compound with its biological activity intact. The above-mentioned enzymes are specific for phosphocholine; other esterases of the signal transduction system would hydrolyze the other phosphoesters *(Methods in Enzymology,* Vol. 201, T. Hunter, Academic Press, NY, Beth Sefton, editor). In this manner, addition of phospholipid is expected to protect compounds from first-pass inactivation in the liver and allow them to reach their sites of action in the blood or in peripheral tissues.

Pharmaceutical agents suitable for use in the present invention include, without limitation, lipophilic compounds that exhibit poor solubility in biological fluids, as well as compounds that are rapidly metabolized in the liver to hippurate, glucuronate, or other derivatives. Non-limiting examples of suitable compounds include those that are not presently utilized in pharmaceutical applications, in particular as orally administrable agents, because of problems with solubility, uptake, and/or metabolism. The only requirements for an agent to be used in the present invention are 1) the presence of a free alcohol functional group to which a phospholipid may be attached, and 2) the susceptibility of the resulting phosphodiester bond to cleavage by phospholipase C, sphingomyelinase or other mammalian esterases.

Examples of pharmaceutical agents suitable for use in the present invention include without limitation steroids, catecholamines such as epinephrine or norepinephrine, prostaglandins such as prostaglandin El or E2, leukotrienes such as leukotriene B4, C4 or D4 and peptides. Peptides for use in the present invention are those which contain serine or threonine and preferably should not be longer than 10-15 amino acid residues in length such as Leutinizing Hormone Releasing Hormone (LHRH) (a 10 amino acid peptide) and its analogues. Preferred starting compounds or pharmacological agents include testosterone (available from Sigma, St. Louis, MO), etiocholanolone (Sigma), estradiol (Sigma), estrone (Sigma) and dehydroepiandrosterone (Sigma). These steroids have only limited activity when administered orally.

In an alternative embodiment of the present invention antibiotics, such as cephalosporin P1, can be conjugated to phospholipids in order to increase its aqueous solubility and decrease it metabolism on the first pass through the liver and excretion on the first pass through the kidney. Non-limiting examples of compounds for use in this

embodiment of the present invention include cephalosporin P1 (isolated as described in Burton et al., Biochem. J. 50:168-174, 1951; Halsall et al., Chem. Comm., pp. 685-687, 1966), fusidic acid (commercially available from Sigma), and helvolic acid (commercially available from Sigma). Use of these antibiotics has been limited because of an inability to development therapeutic serum and tissue levels in recipient mammals and, perhaps, because of the ease of development of resistance. The apparent resistance may be caused by induction of metabolic enzymes as occurs with other steroidal therapeutic agents.

Non-limiting examples of additional substances for use in the present invention containing a free alcohol group include the steroidal substances mentioned above (DHEA, eticholanolone, testosterone, estradiol, estrone, catecholamines, etc.), the antibiotics mentioned above, aglycones including cardiac glycosides, such as digoxigenin (commercially available from Sigma), digitoxigenin (commercially available from Sigma), ouabagenin (commercially available from Sigma) and salicylic acid (commercially available from Sigma).

Presented below is a further list of non-limiting examples of compounds for use in the present invention. Following the name of the compound, presented in parentheses is the number assigned to the compound in the Merck Index, 1996, 12th Edition. Menadiol (5873), Metronidazole (6242), Clindamycin (2414), Pentaerythritol Tetranitrate (7249), Mesalamine (5964), ß-Tocopherol (9632), γ-Tocopherol (9633), δ-Tocopherol (9634), Roxindole (8432), Vitamin E (10159), Styramate (9027), Strophanthidin (9015), Vitamin A (10150), Vitamin D_2 (10156), Vitamin D_3 (10157), Vitamin A_2 (10151), Calcitriol (1681), Diflunisal (3190), Clavulanic Acid (2402), Retinoic Acid (8333), Mazindole (5801).

A compound particularly well-suited for use in the present invention is the cyclic Urea-based HIV-1 protease inhibitor DMP-323 (*J. Med. Chem.* 39:2156-2169, 1996). Due to its low aqueous solubility investigators found that there was variability in the compounds bioavailability upon administration to patients and inconsistent efficacy. Addition of a phospholipid moiety is expected to improve its therapeutic use.

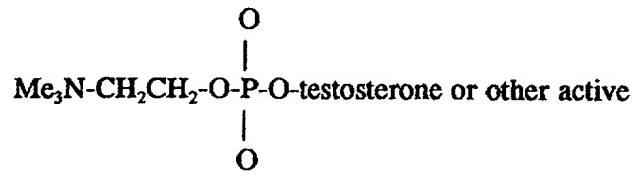
Other compounds well-suited for use in the present invention include aglycones from cardiac glycosides such as digoxigenin, digitoxigenin and ouabagenin (all commercially available from Sigma, St. Louis, MO.).

In addition to increasing the solubility of the above-identified compounds, the primary effect of conjugation to a phospholipid moiety to the following water soluble compounds is expected to be an increased half-life, that is to say, they will be long-acting forms of the parent compounds. Non-limiting examples of such compounds include Isoproterenol (5236), Propranolol (8025), Methyldopa (6132), Epinephrine (3656), Codine (2525), Codine Phosphate (2528), Acetaminophen (45), Aspirin (886).

The conjugated therapeutic agents will be at least ten times more water soluble then the original alcohol. This will increase their bioavailability and decrease their metabolism to, e.g., the 3-glycoside in the case of steroids, which should be a major excretion pathway. The decreased glycoside formation will be caused by the presence of the phosphoester at that site. The derivative is not expected to be active prior to hydrolysis of the phospholipid group. The present inventor has found that lymphocytes have an enzyme on their cell membrane that cleaves phosphocholine from other compounds (for example, sphingomyelin or lecithin) to release phosphocholine and the other ester conjugate (ceramide or diacylglycerol) . The activity of this enzyme is stimulated ten-fold by TGF-α (data not shown). Without wishing to be bound by theory, it is believed that use of phospholipid-conjugated antibiotics of the present invention will lead to high concentrations of active agents at the site of an infection by the following mechanism. Lymphocytes are attracted to the site of an infection or inflammation where they release TGF-$\hat{\alpha}$, which, in turn, stimulates phospholipid hydrolysis in other subtypes. This same process will lead to local release of an active form of the antibiotic from the phospholipid diester conjugate. Because of the response of the enzyme to local concentrations of TGF-α, there should be a correspondingly high local concentration of the antibiotic. This will lead to effective therapy and lower toxicity.

According to the present invention, starting compounds may be converted to phospholipid derivatives using any methods that are known in the art. In one preferred embodiment, phosphocholine (obtainable from Sigma Chemicals, St. Louis, MO) is reacted with a soluble carbodiimide, preferably 1-ethyl-3(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDAC, Sigma) in an active ester condensation reaction. This carbodiimide is preferred because it, similar to phosphocholine, is water-soluble. The active phosphoester intermediate is then reacted with a pharmaceutically active agent to yield the desired phosphocholine ester. The reaction is shown in Example 1 below. Phosphocholine in water is reacted with EDAC to yield the active ester. This

is then reacted with, e.g., testosterone or other biologically active starting compounds etc., to yield the final product



or other active esterification product. The product is expected to be essentially water-soluble and thus easily separated from the starting compound by conventional extraction and/or separation methods e.g. Flash Chromatography, Thin Layer Chromatography, High Performance Liquid Chromatography (HPLC) and the like, as is known to those of ordinary skill in the art.

Alternate methods for synthesis of phosphocholine derivatives include phosphorylation of the steroid, peptide, etc. with DPPP to give a phosphate ester, e.g., testosterone phosphate, which is coupled to choline using EDAC as the complexing agent.

Alternately, the alcohol ("drug") may be reacted with phosphorous oxychloride and the aminoalcohol component added in excess. In this way all of the unreacted phosphorous oxychloride will be used up. The phosphochloride ester intermediate can also be isolated and reacted as a second step with the amino-alcohol component (choline, etc.) . The final products can be purified by HPLC.

The phospholipid derivatized drugs of the present invention are expected to demonstrate enhanced biological activities, increased bioavailability and increased aqueous solubility. For example, etiocholanolone is metabolized by formation of the glucuronide in the liver of a mammal. After oral administration, about 99% of all free etiocholanolone is inactivated on each pass through the liver. When etiocholanolone is orally administered, it is absorbed in the gastrointestinal tract and transported via the portal circulation directly to the liver. Subsequently, only a fraction of a percent of the administered drug is biologically available for function. In contrast, phosphocholine-conjugated etiocholanolone may bind to form Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL) cholesterol and is not expected to be degraded on first passage through the liver. In its phosphocholine-derivatized form, it is believed that about 80% of the etiocholanolone would not be metabolized at each pass. When the phosphocholine moiety is removed by an esterase, such as phospholipase C, sphingomyelinase, etc., then the parent compound will be available for binding and function in the target tissue.

Glucuronidation would only occur on its return to the liver after removal of the phosphocholine moiety.

The phospholipid-conjugated compounds of the present invention may be administered therapeutically by any route known in the art, e.g., orally, intravenously, intramuscularly, subcutaneously, by inhalation or in aerosol form, and topically. The present invention is particularly applicable to compounds that, in their unconjugated state, cannot be effectively administered by the oral route.

The phospholipid-conjugated compounds of the present invention can be tested for efficacy as follows. A starting compound, and its phospholipid derivative, may be administered by any of the above routes to a test animal, e.g., rat, mouse, rabbit, guinea pig, and the like. Serum samples are then collected at increasing times after administration, and the levels of the starting and conjugated compound are assayed and compared. It will be understood by those skilled in the art that the method of assay will depend upon the starting compound. In the case of steroids or peptides, High-Performance Liquid Chromatography, Thin-Layer Chromatography, or immunoassay may be used to quantify serum levels. When the starting compounds are gonadal steroids, it may also be necessary to gonadectomize the test animals prior to drug administration, so as to suppress endogenous production of the test compound. Successful compounds are those whose serum level is increased significantly by administration of the phospholipid derivative relative to administration of the starting compound or by their ability to reach therapeutically significant serum levels when administered by an alternate route, e.g. orally.

In a second phase, the starting compound and its phospholipid derivative will be administered to test animals, and the physiological effect of the compounds assayed over time. For example, for etiocholanolone and its phospholipid derivative(s), rate of weight gain and changes in basal metabolic rate are measured. Estradiol, estrone and their phosphocholine derivatives will be administered by gavage to ovariectomized mice or rats and changes in uterine weight, breast development and estradiol blood levels will be measured. Testosterone and its phosphocholine derivative will be administered orally to castrate mice or rats and changes in seminal vesicles, prostate size, and levator and muscle will be determined. Theophylline and its phosphocholine derivatives will be given orally to rats and the blood levels over the next 6 hours will be determined. From these tests, the degree to which the phospholipid derivatives are more potent than the

underivatized parent compound will be determined, i.e., the same response will be achieved with a smaller dose of the derivatized compound than the parent compound. This will be a measure of greater potency. Successful compounds are those whose functional endpoints are significantly lower for phospholipid derivatives than for the starting compounds.

In a preferred embodiment of the present invention, testosterone is converted to testosterone-17-phosphocholine, estrone is converted to estrone-3-phosphocholine and estradiol is converted to estradiol-3-phosphocholine or estradiol-17-phosphocholine. In like manner, theophylline is converted to theophylline phosphocholine. These compounds will frequently be given as replacement therapy for various hormone deficiencies and as pharmacological therapies in other cases. Theophylline is given to treat asthma, estradiol is administered to treat osteoporosis, etiocholanolone is given as a haemapoetic agent, to promote weight loss and to reduce diabetic blood sugar levels. Similar derivatives could also be used to provide enhanced levels of epinephrine.

The present invention also provides pharmaceutical formulations and dosage forms comprising the phospholipid-derivatized drugs of the present invention. The pharmaceutical formulations of the present invention may also include, as optional ingredients, pharmaceutically acceptable vehicles, carriers, diluents, solubilizing or emulsifying agents, and salts of the type well known to those of ordinary skill in the art.

The phospholipid-derivatized drugs of the present invention can be incorporated into pharmaceutical formulations to be used to treat mammals. Pharmaceutical formulations comprising the phospholipid-conjugated drugs of the present invention as at least one of the active ingredients, would in addition optionally comprise pharmaceutically-acceptable carriers, diluents, fillers, salts and other materials well-known in the art depending upon the dosage form utilized. For example, preferred parenteral dosage forms may comprise a sterile isotonic saline solution, 0.5 N sodium chloride, 5% dextrose and the like. Methyl cellulose or carboxymethyl cellulose may be employed in oral dosage forms as suspending agents in buffered saline or in cyclodextran solutions to enhance solubility.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose or dosage form need not in itself constitute an effective amount for the various usages of the phospholipid-derivatized drugs of the present

invention since the necessary effective amount can be reached by administration of a plurality of such dosage forms.

The following examples are intended to further illustrate the present invention without limiting it thereof.

EXAMPLE 1 : SYNTHESIS OF PHOSPHOCHOLINE DERIVATIVES

Method 1

Phosphocholine (Sigma) (0. 1 mol) is stirred in pyridine (Fisher, VWR) (100 ml) with 0.1 mol of morpholine (Sigma) and 0.1 mol of DDC (Sigma) for 6 hours under nitrogen or argon. At this point the reaction complex is stirred while 0.1 mol of steroid (etiocholanolone, estradiol, testosterone) are added. After stirring for an additional 3 hours the reaction mixture is diluted with 1 liter of ice water. The insoluble N,N' dicyclohexylurea is removed by filtration and the aqueous fraction is extracted with 4 X 0.5 volumes of ethyl acetate. The ethyl acetate is washed with saturated brine (0.1 vol) to remove the pyridine and dried over sodium sulfate. The solvent is removed by filtration and the product isolated by LH-20 column chromatography or by preparative HPLC.

Method 2

Phosphocholine (0.1 mol), steroid (0.1 mol) as above and DCC (0.12 mol) are stirred in 100 ml of pyridine (VWR) at 80° for 6 hours under nitrogen. The solution is diluted with 600 ml of water and processed as described above.

Method 3

Testosterone or other steroid, prostaglandin, etc. (0.1 mol) is reacted with $POCl_3$ in pyridine to yield the steroid phosphate. This product after drying in pyridine will then be reacted with 0.1 mol of EDAC at a rate just sufficient to maintain the pH at 7.0. The product is then purified as described above.

The compounds will then be analyzed by HPLC to determine purity of the reaction product, by NMR to verify the structure and by UV and IR spectra to determine their identity. Treatment with a phosphodiesterase will then be used to cleave the diester to further establish the structural identity.

EXAMPLE 2: PHARMACOKINETICS OF TESTOSTERONE AND ITS PHOSPHOCHOLINE DERIVATIVE

The phosphocholine derivatives of testosterone (about 5 mg) is dissolved in 20 ml of buffered saline or in 20 ml of 40% cyclodextran in saline and given orally to human volunteers. Alternatively, testosterone (5 mg) is suspended in a carboxymethyl cellulose suspending media, vortexed and then given orally. Blood samples will be taken at 30, 60, 120, 240, 360 and 720 minutes post-administration and collected in green top tubes. The blood samples are centrifuged and the plasma collected and stored as aliquots in microfuge tubes. The samples are then analyzed for testosterone in duplicate using a standard RIA kit (Diagnostics Products Corp., Tarzana, CA).

EXAMPLE 3: MEASUREMENT OF BIOACTIVITY OF PHOSPHOCHOLINE DERIVATIVES

The bioactivity of orally administered estradiol and estradiol phosphocholine will be determined in ovariectomized mice or rats. In addition, other animals will be briefly anesthetized and the steroid phosphocholine derivative or the free steroid will be administered intraperitoneally (IP). After 2 days the animals are sacrificed and the 4th and 9th inguinal breast tissue will be isolated. At the same time the uteri will be isolated and weighed. It is expected that the phosphocholine derivatized steroid will be more active than the parent compound when administered orally and by IP injection.

Estradiol and its phosphocholine derivative will also be administered by gavage to ovariectomized mice or rats and changes in uterine weight, breast development and estradiol blood levels will be measured. Estradiol will be measured with an RIA kit from Diagnostics Products Corp. (Tarzana, CA).

Testosterone and its phosphocholine derivative will be administered orally to castrate male mice or rats and changes in seminal vesicles, prostate size, and levator ani muscle will be determined. Testosterone blood levels will also be measured by RIA using a kit from Diagnostics Products Corp. (Tarzana, CA). The compounds will also be characterized by UKV. Responses will also be measured after IP injection.

Theophylline and its phosphocholine derivatives will be given orally to rats and the blood levels of theophylline will be measured over the next 6 hours using an RIA kit (Diagnostics Products Corp., Tarzana, CA).

From these tests, the degree to which the phosphocholine derivatives are more potent than the underivatized parent hormone can be determined; i.e., the same response will be achieved with a smaller dose of the derivatized compound than the 25 parent compound. This will be a measure of greater potency.

EXAMPLE 4: SYNTHESIS OF DEHYDROEPIANDROSTERIONE (DHEA)-PHOSPHOCHOLINE DERIVATIVE

A dehydroepiandrosterone(DHEA)-phosphocholine derivative was synthesized as follows: 1 mg of phosphocholine (calcium salt; Sigma Chemical, St. Louis, MO) was dissolved in 0.5 ml of formamide (Cat # S-7503; Lot # 55HO257; Sigma Chemical) and 0.5 ml of pyridine (Cat # P-4036; Lot # 55Hl489; Sigma Chemical). 0.025 mCi of (1,2,6,7 ^{3}H(n)-Dehydroepiandrosterone (Cat # NET814; Lot # 3146097; 89.2 Ci/mmol; Dupont, NEN Products, Boston, MA) in 0.025 ml of ethanol was added. The reaction was catalyzed by the addition (as the dry solid) of 5 mg of dicyclohexylcarbodiimide (Cat # D-3129; Lot # 34hO647; Sigma Chemical). The reaction mixture was incubated overnight at room temperature. In the morning, 9 ml of water was added and the mixture extracted 3 times with 10 ml portions of benzene. The benzene extracts were combined and aliquots of both phases were counted in a scintillation counter. The results are set forth below:

Aqueous Phase	10,729 cpm (O.O1ml)
Benzene Phase	1,121 cpm (O.Olml)

The aqueous layer was re-extracted with benzene. The second benzene extraction yielded 272 cpm (0.01 M1) as a confirmation.

Free DHEA starting material would have been extracted quantitatively into benzene with this protocol. The observation that the reaction product remains in the aqueous phase confirms its increased hydrophilic characteristics.

Example 5: DHEA-3-PHOSPHOCHOLINE: SYNTHESIS AND BIOACTIVITY

DHEA-phosphocholine (DHEA-PC) was synthesized by sequential reaction of DHEA, choline, and water with phosphorous oxychloride. The synthetic product had the same HPLC retention time and the same mass-spectrum as did the endogenous, actual compound. It was hydrolyzed by neutral sphingomylenase, but not by acidic

sphingomylenase. When human serum extracts were analyzed, mass fragments were detected at the same retention time as synthetic material. When DHEA-PC was administered to mice, it potentiated dinitrochlorobenzene-induced sensitization as detailed below.

Example 6: DHEA-PC POTENTIATES DNCB-INDUCED IMMUNOLOGICAL SENSITIZATION

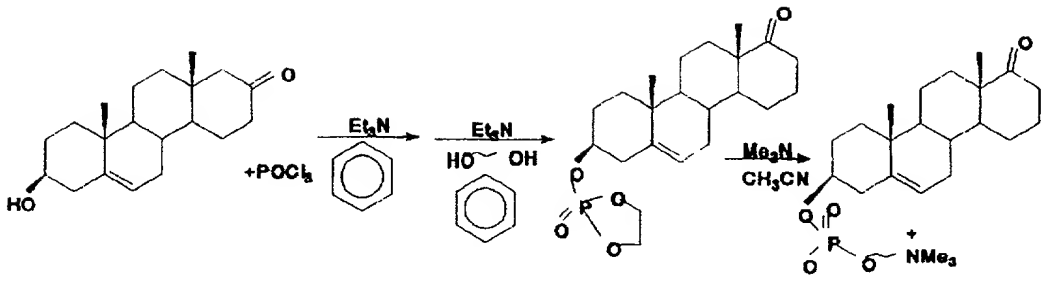
The effects of DHEA-PC on cutaneous contact hypersensitivity was studied. In this study, mice (Balb/c) were immunologically challenged with DNCB (2% in ethanol) applied to a 2 cm area on the back. The steroid was injected subcutaneously (100 μg/day/mouse) throughout the twelve-day study period. Ears were rechallenged with DNCB (1% in ethanol) on days 7-12 and swelling was measured daily in order to evaluate the effect on the immune system.

During days 7-12, DHEA-PC enhanced the cutaneous hypersensitivity immune response similar to native steroids (DHEA and DHEA-sulfate). The response to these three hormones was not suppressed by dexamethasone even though, when administered by itself, dexamethasone suppressed the immune response below the control. This shows that all three hormones induced a similar, high level response.

Example 7: NOVEL PHOSPHOCHOLINE SYNTHETIC METHOD

DHEA (I) (82.0g, 0.284 mol, Steraloids, Inc., Wilton, NH) was dissolved in a 5 L, 3 necked, round bottom flask in dry benzene (1.5 L, Fisher, Pittsburgh, PA). Gentle heating was applied to facilitate the process. Triethylamine (30.3g, 41.6 mL, 0.30 mol, Aldrich, Milwaukee, WI) was added all at once. After the reaction was cooled down to room

temperature, oxyphosphorus trichloride (43.6 g, 26 mL, 0.284 mol, Fluka, Ronkonkoma, NY) was added in one portion. The mixture was stirred under nitrogen overnight (12 hours). The precipitate was filtered off via canula transfer under nitrogen, and washed once with dry benzene (300 mL). To the combined clear benzene solution was added ethylene glycol (18.6 g, 0.30 mol, Aldrich) and triethylamine (61 g, 0.60 mol, Aldrich). The mixture was stirred rapidly for 16 hours at room temperature. Thin layer

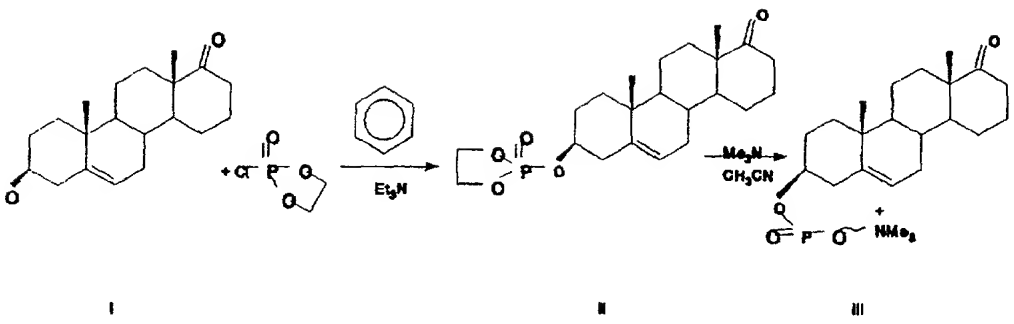


I II III

chromatography (TLC) (Silica gel,

developed with ethyl acetate, Fisher) showed almost complete conversion. The newly formed precipitate was separated on a Buchner funnel and washed three times with hot dry benzene (800 mL total). The combined filtrates were evaporated to dryness on a rotary evaporator (Buchi, Fisher). The intermediate (II) was a white solid and used for the next step without further purification. An additional amount of the intermediate (II) was obtained from the solid retained by the Buchner funnel by resuspension in water and vacuum filtration. The combined collected solid was air dried. The overall yield of the crude intermediate (II) was virtually quantitative (110 g).

The crude II (2.9 g) was suspended in acetonitrile (25 mL, Fisher Chemicals). While the mixture was stirred at 50-60°C with the pressure maintained through a balloon, trimethylamine (Aldrich) was introduced as the gas. After the conversion was complete, as indicated by TLC analysis, the mixture was vacuum filtered, washed repeatedly with acetonitrile and then air dried. The yield was 75% (2.5 g). LC-mass spectroscopy (Micromass, Beverly, MA) showed a major peak at Rf=9.8 min with mass of 454 daltons (M+H), as predicted. DHEA (8.75g, 0.031 mol) was dissolved in benzene and triethylamine (4.45 mL) was added. 2-choro-1,3,2-dioxaphospholane-2-oxide (4.54g, 0.032 mol, Aldrich) was then added at room temperature. The reaction mixture was stirred until complete conversion of DHEA to II occurred. The reaction was monitored by TLC (silica gel, ethyl acetate). After filtration, the solid was washed with dry benzene. The combined benzene solution was concentrated to give a white solid (II) and



I II III

used without further purification.

A sample of II (0.75 g) prepared as above was suspended in acetonitrile (10 mL) and stirred with heating. Trimethylamine was introduced as a gas while the pressure was regulated with a balloon attached to one of the necks of the flask. When TLC (silica gel, ethyl acetate) showed the disappearance of II, the addition of gas was stopped. The product (III) was collected by vacuum filtration, washed with additional acetonitrile and air dried. The yield was 0.72g (83%).

WHAT IS CLAIMED IS:

1. A method for increasing the aqueous solubility of a pharmaceutically active agent, comprising the steps of

conjugating said agent to a phospholipid moiety, wherein said phospholipid moiety is selected from the group consisting of phosphoserine, phosphotyrosine, phosphoethanolamine, n-monoalkyl-phosphoethanolanolamine and N, N-dialkyl-phosphoethanolamine and

recovering said pharmaceutically active agent conjugated to said phospholipid.

2. The method of claim 1, wherein said agent is selected from the group consisting of a steroid, peptide, prostaglandin, catecholamine, and a leukotriene.

3. The method of claim 1 wherein said agent is an antibiotic selected from the group consisting of cephalosporin P1, fusidic acid and helvolic acid.

4. The phospholipid conjugated pharmaceutically active agent produced by the method of claim 1.

5. A pharmaceutical formulation comprising a phospholipid-conjugated active agent wherein said agent is selected from the group consisting of testosterone, estrone, estradiol, etiochaolanolone, and dehydroepiandosterone and a pharmaceutically-acceptable carrier or diluent wherein said phospholipid is selected from the group consisting of phosphoserine, phosphotyrosine, phosphoethanolamine, n-monoalkyl-phosphoethanolanolamine and N, N-dialkyl-phosphoethanolamine.

6. A pharmaceutical formulation for treating a mammal suffering from asthma comprising an isolated phospholipid derivative of theophylline and a pharmaceutically acceptable carrier or diluent wherein said phospholipid is selected from the group consisting of phosphoserine, phosphotyrosine, phosphoethanolamine, n-monoalkylphosphoethanolanolamine and N, N-dialkyl-phosphoethanolamine.

7. A pharmaceutical formulation comprising an isolated phospholipid derivative of an antibiotic selected from the group consisting of cephalosporin Pl, fusidic acid and helvolic acid, and a pharmaceutically acceptable carrier or diluent.

8. A pharmaceutical formulation comprising a phospholipid-conjugated pharmaceutically active agent wherein said agent is selected from the group consisting of digoxigenin, digitoxigenin, ouabagenin and salicylic acid, and a pharmaceutically acceptable carrier or diluent.

9. A pharmaceutical formulation comprising a biologically-active phospholipid-conjugated pharmaceutically active agent wherein said agent is selected from the group consisting of Menadiol, Metronidazole, Clindamycin, Pentaerythritol Tetranitrate, Mesalamine, ß-Tocopherol, γ-Tocopherol, δ-Tocopherol, Roxindole, Vitamin E, Styramate, Strophanthidin, Vitamin A, Vitamin D_2, Vitamin D_3, Vitamin A_2, Calcitriol, Diflunisal, Clavulanic Acid, Retinoic Acid, and Mazindole and a pharmaceutically acceptable carrier or diluent.

10. A pharmaceutical formulation comprising a phospholipid-conjugated derivative of DMP-323 and a pharmaceutically acceptable carrier or diluent.

11. A pharmaceutical formulation comprising a phospholipid-conjugated pharmaceutically active agent wherein said agent is selected from the group consisting of Isoproterenol, Propranolol, Methyldopa, Epinephrine, Codeine, Codeine Phosphate, Acetaminophen, and Aspirin, and a pharmaceutically acceptable carrier or diluent.

12. A composition of matter comprising an isolated phospholipid derivative of an antibiotic selected from the group consisting of cephalosporin Pl, fusidic acid and helvolic acid.

13. A composition of matter comprising a phospholipid-conjugated pharmaceutically active agent wherein said agent is selected from the group consisting of digoxigenin, digitoxigenin, ouabagenin and salicylic acid.

14. A composition of matter comprising a biologically-active phospholipid-conjugated pharmaceutically active agent wherein said agent is selected from the group consisting of Menadiol, Metronidazole, Clindamycin, Pentaerythritol Tetranitrate, Mesalamine, ß-Tocopherol, γ-Tocopherol, δ-Tocopherol, Roxindole, Vitamin E, Styramate, Strophanthidin, Vitamin A, Vitamin D_2, Vitamin D_3, Vitamin A_2, Calcitriol, Diflunisal, Clavulanic Acid, Retinoic Acid, and Mazindole.

15. A composition of matter comprising a phospholipid-conjugated derivative of DMP-323.

16. A composition of matter comprising a phospholipid-conjugated pharmaceutically active agent wherein said agent is selected from the group consisting of Isoproterenol, Propranolol, Methyldopa, Epinephrine, Codine, Codine Phosphate, Acetaminophen, and Aspirin.

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Includes Reference to PCT International Applications)

ATTORNEY DOCKET NUMBER
5412/1E887US2

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed for and which a patent is sought on the invention entitled:

Phosphocholine Linked Prodrug Derivatives

the specification of which (check only one item below):

[] is attached hereto.

[] was filed as United States application

Serial No. _____

on _____

and was amended

on _____ (if applicable).

[X] was filed as PCT international application

Number **PCT/US00/04140**

on **16 February 2000**

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119
US	60/120,483	18 February 1999	[X] YES [] NO
PCT	PCT/US00/04140	16 February 2000	[] YES [X] NO
			[] YES [] NO
			[] YES [] NO
			[] YES [] NO

US

Combined Declaration for Patent Application and Power of Attorney (Continued)
(Includes Reference to PCT International Applications)

ATTY'S DOCKET NUMBER
5412/11887US2

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S APPLICATIONS		STATUS (Check one)		
U.S APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U S SERIAL NUMBER ASSIGNED (if any)			

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. Morris Relson #15,108, Gordon D. Coplein #19,165, William F. Dudine, Jr. #20,569, Michael J. Sweedler #19,937, S. Peter Ludwig #25,351, Paul Fields #20,298, Joseph B. Lerch #26,936, Melvin C. Garner #26,272, Ethan Horwitz #27,646, Beverly B. Goodwin #28,417, Adda C. Gogoris #29,714, Martin E. Goldstein #20,869, Bert J. Lewen 19,407, Henry Sternberg #22,408, Peter C. Schechter #31,662, Robert Schaffer #31,194, David R. Francescani #25,159, Robert C. Sullivan, Jr. #30,499, and Joseph R. Robinson #33,448, Walt Thomas Zielinski #18,902, Eugene L. Szczecina, Jr. #35,029

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3	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE 20 Sept 2001	DATE	DATE

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US

Combined Declaration for Patent Application and Power of Attorney (Continued) (Includes Reference to PCT International Applications)	ATTY'S DOCKET NUMBER 5412/11887US2

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S APPLICATIONS		STATUS (Check one)		
U.S APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

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SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
DATE	DATE 10/10/01	DATE

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